Patent
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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application of

Marshall S. Horwitz et al

Serial No.: 09/132,231

Filed: August 11, 1998

For: METHOD FOR PRODUCING NOVEL DNA SEQUENCE WITH BIOLOGICAL ACTIVITY

Group Art Unit: 1632

Examiner: J. Brusca

DECLARATION PURSUANT TO 37 CFR 1.131

Honorable Commissioner of Patents & Trademarks
Washington, D.C. 20231

Sir:

I, Dr. Marshall S. Horwitz, declare as follows:

1. I am the first-named inventor of the invention disclosed and claimed in the above-identified patent application, which is a continuation of Application No. 08/316,415 filed on September 30, 1994, which is a continuation of Application No. 08/105,108, filed on August 11, 1993, which is a continuation of Application No. 07/881,607, filed on May 12, 1992, which is a continuation of Application No. 07/368,674, filed on June 19, 1989, which is a continuation-in-part of Application No. 06/887,070, filed July 17, 1986.

2. The experimental work performed by me at University of Washington in the laboratory of Dr. Larry Loeb involving the selection of random nucleotide and peptide sequences having a desired biological activity was routinely entered in my laboratory notebooks. The date, typically appearing at the top of the page, represents the date that the work recorded on that page was commenced. (The dates have been redacted.) Each notebook page is numbered in either the upper right hand or left hand corner. Attached to this Declaration is a true copy of relevant pages from one of my notebooks. In this notebook, there were two pages each for each number, i.e., two page 001's, two page 002's, etc., therefor, in some cases the page numbers are referenced along with the letters "A" or "B" to indicate whether the relevant page was the first or second entry in the notebook, respectively.

3. It is my understanding that the Examiner has suggested that U.S. Patent No. 5,866,363 to Pieczenik may be prior art against my application if it is entitled to the filing date of the grand-parent application (August 28, 1985). It is also my understanding that, if I can demonstrate that I made my invention prior to the Pieczenik August 28, 1985, filing date, that this patent could no longer be applied as prior art against my invention.

4. Prior to August 28, 1985, I conceived of, discussed with colleagues and conducted experiments in the United States, which demonstrated the feasibility of a method for identifying a functional DNA sequence which provides a desired biological activity comprising

(a) providing a means for detecting said desired biological activity; (b) synthesizing a mixed population of random DNA sequences; (c) introducing a plurality of random DNA sequences into a population of cloning vectors (into a structural gene in the vector) to obtain a plurality of cloning vectors containing random DNA sequences; (d) introducing said cloning vectors into suitable host cells; (e) expressing said cloning vectors in said host cells; and (f) screening said host cells using said means for detecting the desired biological activity under conditions which allow detection of one or more host cells comprising vectors which comprise a functional DNA sequence which provides the desired biological activity. The experimental methods entailed making a population of recombinant vectors comprising an oligonucleotide population encoding a plurality of random peptide sequences which were expressed in host cells for the purpose of identifying from the mixed population of randomly generated DNA sequences a functional DNA sequence which provides a desired biological activity. These experiments are memorialized in notebook pages attached to this Declaration.

5. For instance, prior to August 28, 1985, I tested the activity of the terminal deoxynucleotidyl transferase (TdT) enzyme to be used in the synthesis of a random population of nucleotides. The experiments are recorded in at pages 002-008B (actual dates masked). For instance, as described on page 003, three batches of enzyme were tested (1 λ, .1 λ, .01 λ and .001 λ) in a "TdT assay" using poly(A) and dGTP. As recorded on page 008B, the reaction

using poly(A)$_{100}$ as a positive control worked, suggesting the DNA (i.e., the vector) needed further refinement.

6. Prior to August 28, 1985, I then optimized the quantities of vector and deoxynucleotides to be used in the TdT reaction by experimenting with the relative molar concentrations of vector and either dGTP or dTTP alone. These experiments are recorded in my notebook at pages 008B-016A. For instance, as described on page 012A, that particular assay tested 1/10 and 1/100 of the molar concentration of vector used in the previous test. "This effectively [raised] the relative dGTP/3'OH ratio by a factor of 10." Similarly, as described on page 013A and 014A, experimenting with different quantities of vector, i.e., 1μg/λ and .1 μg/λ, in a TdT assay with ^{32}P-labeled dTTP yielded a population with 488 T/ 3' end (of vector) and 220 T/ 3' end, respectively, as measured by the quantity of radioincorporation.

7. Prior to August 28, 1985, I conducted TdT assays using all four dNTPs, thereby creating a random population of nucleotide sequences attached to the vector pUC13. Some of these experiments are recorded at pages 016B-018 (actual dates masked). As described on page 016B, the relative quantities of each nucleotide incorporated were calculated based on the amount of radioactivity incorporated during two separate reactions, one in which ^{32}P-labeled dTTP and ^{3}H-labeled dGTP were used, and one in which ^{32}P-labeled dCTP and ^{3}H-labeled

dATP were used. As reported on page 017, the first random pool of sequences was predicted to contain about 34.4% A, 15.8% C, 36.3% G and 13.5% T, with the average length of random addition to the vector being 691 nucleotides. And as indicated on page 018B, the samples were loaded onto a sequencing gel which was run by a technician, "Clair" (Clair Maxwell), for the purpose of verifying the random nature of the sequences.

8. Prior to August 28, 1985, I calculated the probability of stop codon appearance based on the frequency of incorporation of each of the four nucleotides and experimentally adjusted the relative amounts of dNTPs to be employed in the TdT assay to affect stop codon bias. These calculations and experiments are reported in the attached notebook pages 019A-025A. For instance, as described on page 019A, if the termination codons are the triplets TAA, TAG and TGA, then the probability of any random triplet being a stop codon can be calculated by the following equation $(T)(A^2) + 2(T)(A)(G)$, where the letters in the equation represent the percentage probability of incorporating that particular nucleotide at the given position in the triplet. Using the probabilities calculated in my initial experiments, i.e., 34.4% A, 15.8% C, 36.3% G and 13.5% T, respectively, the probability of incorporating a stop codon in any given position would be $(.135)(.344)^2 + (2)(.135)(.363)(.344)$, or .04969. Based on this probability, I figured that the fraction of unterminated nucleotide sequences in a population having, on average, 100 nucleotides would be only about .006. I then inserted different probabilities for incorporating each of the nucleotides into the equation to determine

which probabilities would yield a more acceptable probability of unterminated sequences, and found that lowering the probability of incorporating an A (to 5- 15%) and raising the probability of incorporating a C or T would yield a more favorable fraction of unterminated sequences (pages 019A-022B; actual dates masked). I then calculated the concentration of each dNTP to use in the reaction based on ratio of the desired incorporation probability and the actual incorporation probability observed in the initial experiments performed with all four dNTPs (page 019B, 022B), considering as well how the resulting distribution of amino acids would be affected (page 021B and 025A).

9. Prior to August 28, 1985, I scaled up the TdT reaction to effectuate a large scale random tailing reaction, and conducted experiments to determine the optimum amount of enzyme to be used in the tailing reaction. These experiments are reported in my notebook at pages 025B-064B.

10. Prior to August 28, 1985, I conducted experiments to generate functional vectors comprising the TdT-generated random sequences by allowing the DNA from the tailing reaction to anneal, filling in the gaps with dNTPs using Klenow polymerase I, and ligating the vectors with DNA ligase (notebook page 065). The ligated pool was then transformed into *E. coli* JM83 using ampicillin resistance to select for transformed colonies, and X-gal was used to signal the presence of inserted DNA in the transformed vectors (pages 066B-068A).

(Disruption of the beta-galactosidase gene in pUC19 by insert DNA would result in white transformant colonies on medium containing the indicator X-gal instead of blue colonies). Several "white" colonies grew on selection media containing ampicillin and X-gal, indicating that these transformants had potentially received pUC19 vector containing insert DNA which disrupted the beta-galactosidase gene (page 069B). Several transformants were grown in liquid culture, and vectors were isolated from transformants to verify the presence of insert DNA following restriction digestion (pages 070A-070B, 071, 072A, 073). Random inserts of various sizes were identified.

11. Prior to August 28, 1985, I screened transformants containing random sequences of DNA for those which encoded random polypeptides which provided antibiotic resistance. In order for selection to be successful, the host cell into which vectors were transformed could not be naturally resistant to the antibiotic used to screen the transformant colonies. Likewise, vector without insert DNA should not confer antibiotic resistance. Therefor, random transformant populations were screened at the same time as cells transformed with pUC19 without insert for cells which demonstrated resistance to a variety of antibiotics (pages 074B-075A). As reported on page 075A of my notebook, transformants from the random insert population were observed growing near both sulfamethoxazole trimethoprim (SXT) and celfoxitin (FOX 30) antibiotic disks, whereas the corresponding pUC19 (no insert) transformation mix produced no single colonies in the zone around the

disks. In addition, a single transformant colony was isolated from a tetracycline plate several days after the plates were inoculated (page 076B). Individual transformant colonies were incubated into liquid broth and cultured overnight for plasmid isolation (page 078A).

12. I knew at the time that I isolated vectors having random insertions of various sizes from host cells that it would be possible to generate and screen a random library of nucleic acid sequences for those which provided a desired biological function. The experiments I completed prior to August 28, 1985, demonstrated the feasibility of a method whereby functional DNA sequences could be identified by (a) providing a means for detecting a desired biological activity; (b) synthesizing a mixed population of random DNA sequences; (c) introducing a plurality of random DNA sequences into a population of cloning vectors to obtain a plurality of cloning vectors containing random DNA sequences; (d) introducing said cloning vectors into suitable host cells; (e) expressing said cloning vectors in said host cells; and (f) screening said host cells using said means for detecting the desired biological activity under conditions which would allow detection of one or more host cells comprising vectors which comprise a desired functional DNA sequence if such a sequence was present.

13. That I further declare that all statements made herein of my knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application and any patent issuing therefrom.

June
6/11/01
Date

Marshall S. Horwitz
Dr. Marshall S. Horwitz